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Lumbar transplant of neurons genetically modified to secrete brain-derived neurotrophic factor attenuates allodynia and hyperalgesia after sciatic nerve constriction

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Abstract

Chronic delivery of anti-nociceptive molecules by means of cell grafts near the pain processing centers of the spinal cord is a newly developing technique for the treatment of neuropathic pain. The rat neuronal cell line, RN33B, derived from E13 rat brainstem raphe and immortalized with the SV40 temperature-sensitive allele of large T antigen (tsTag), was transfected with rat brain-derived neurotrophic factor cDNA (BDNF), and the BDNF-synthesizing cell line, 33BDNF.4, was isolated. The 33BDNF.4 cells synthesized mature BDNF protein at permissive temperature (33°C), when the cells were proliferating, and during differentiation at non-permissive temperature (39°C) in vitro. The bio-active BDNF protein was also secreted by the cells during both growth conditions, as measured by ELISA analysis of BDNF content and secretion. The bio-activity of the BDNF in 33BDNF.4 cell conditioned media was assessed by neurite outgrowth from E15 dorsal root ganglion (DRG) cultures. A control cell line, 33V1, transfected with the vector alone, did not synthesize or secrete any significant BDNF at either growth condition. Both cell lines were used as grafts in a model of chronic neuropathic pain induced by unilateral chronic constriction injury (CCI) of the sciatic nerve. Pain-related behaviors, including cold and tactile allodynia and thermal and tactile hyperalgesia, were evaluated after CCI in the affected hindpaw. When 33BDNF.4 and 33V1 cells were transplanted in the lumbar subarachnoid space of the spinal cord 1 week after CCI, they survived greater than 7 weeks on the pia mater around the spinal cord and the 33BDNF.4 cells continued to synthesize BDNF in vivo. Furthermore, the tactile and cold allodynia and tactile and thermal hyperalgesia induced by CCI was significantly reduced during the 2–7 week period after grafts of 33BDNF.4 cells. The maximal effect on chronic pain behaviors with the BDNF grafts occurred 2–3 weeks after transplant and the anti-nociceptive effects of the BDNF cell grafts was permanent. Transplants of the control 33V1 cells had no effect on the allodynia and hyperalgesia induced by CCI and these cells did not synthesize BDNF in vivo. These data suggest that a chronically applied, low local dose of BDNF supplied by transplanted cells near the spinal dorsal horn was able to reverse the development of chronic neuropathic pain following CCI. The use of neural cell lines that are able to deliver anti-nociceptive molecules, such as BDNF, in a model of chronic pain offers a novel approach to pain management and such 'biologic minipumps' can be developed for safe use in humans. © 2000 International Association for the Study of Pain. Published by Elsevier Science B.V. All rights reserved.

Keywords: CCI; Neural cell lines; Transplantation; Brain-derived neurotrophic factor; Cellular minipumps

1. Introduction

The mechanisms for the induction and development of neuropathic pain and its relation to spinal nociceptive events following nerve injury has focused on the imbalance of excitatory and inhibitory systems as a likely mechanism (Dickenson et al., 1997). Persistent pain is thought to be mainly the result of the loss or down-regulation of the inhibitory γ-amino butyric acid (GABA) dorsal horn interneurons (Kingery et al., 1988; Yaksh, 1989; Hao et al., 1992; Zhang et al., 1994a; Stiller et al., 1995), increased release of excitatory neurotransmitters, such as glutamate, and up-regulation of glutamate receptors (Harris et al., 1996), with accompanying changes in intracellular Ca^{2+} following nerve injury. However, a role for endogenous neurotrophins in the dorsal horn and dorsal root ganglion (DRGs) in the development of chronic pain has only been recently appreciated (Millan, 1999), with the application of nerve growth factor (NGF) and neurotrophic factor-3 (NT-3) able to increase sensory neuron survival after axotomy (Ljunberg

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et al., 1999). Another neurotrophin, BDNF, has been recognized to date for its survival effects on spinal motorneurons (Novikova et al., 1997) after injury. However, BDNF is a potent survival and differentiation factor for many developing and injured neuronal systems (Ohsawa et al., 1993), including CNS GABAergic (Hyman et al., 1994) and serotonergic (5HT) neurons (Martin-Iverson et al., 1994). BDNF is anterogradely transported by DRG C-fiber afferents (Zhou and Rush, 1996) to their terminal targets in laminae I and II, and to a lesser extent laminae IV/V and laminae X (Cho et al., 1998), where BDNF is concentrated at synaptic terminals. However, the ability to alter BDNF levels with chronic delivery of exogenous neurotrophic factors such as BDNF to the CNS remains problematic as they will not cross the blood brain barrier and must be directly injected or chronically infused. Genetically-engineered fibroblasts, which function as biologic minipumps and which secrete BDNF (Kim et al., 1996) have been used for neural regeneration and recovery after traumatic injury, but such potentially mitotic cells are not appropriate for subdural spinal transplant. Chronic delivery of both BDNF and 5HT from bio-engineered neuronal cell line subdural grafts, functioning as cellular minipumps after peripheral nerve injury (Eaton et al., 1997), was able to attenuate the altered sensory behaviors in neuropathic pain, and suggested that cell transplants that supplied BDNF alone might directly affect the reversal of those behaviors.

Neural transplantation is a potential therapeutic approach to restore function and enhance recovery after injury or neurodegenerative disease and provide analgesia (Czech and Sagen, 1995). Transplants of primary adrenal medullary cells that release peptides and neurotransmitters have offered a new direction in the treatment of chronic pain (Winnie et al., 1993). Transplantation of immortalized cell lines genetically-modified to release neuroactive anti-nociceptive molecules in chronic pain (Eaton et al., 1997, 1999b) and to up-regulate neurotransmitter synthesis (Eaton and Whittemore, 1996; Eaton et al., 1996) offers a renewable source of cells that can act as cellular minipumps able to respond to the microenvironment of the cord. This approach should reduce or eliminate side effects associated with large doses of pharmacologic agents required for centrally-acting pain-reducing agents.

Based on these results, we proposed that a chronic BDNF-secreting cell transplant could be used to alleviate chronic neuropathic pain. In the present study, we describe the alleviation of tactile and cold allodynia and thermal tactile hyperalgesia induced after the chronic constriction injury (CCI) of the sciatic nerve by transplantation of BDNF-synthesizing and -secreting cells near the lumbar spinal cord. Preliminary results of these data have recently been reported (Cejas et al., 1999) and it is expected that this approach to chronically deliver BDNF into the CNS will provide a useful model for the creation of similarly-derived human cell lines and their therapeutic use for chronic pain.

2. Materials and methods

2.1. Transfection of BDNF gene and amplification of clonal cell lines

The rat BDNF construct was provided by Paul J. Isacson, Mayo Clinic (Jacksonville, FL). The cDNA encoding rat BDNF has a unique *Nhe*I site six bases 5′ to the initiation methionine and a unique *Bam*HI site ten bases 3′ to the stop codon. This 764 bp fragment was cloned into similar sites in the eukaryotic expression vector pCEP4 with the restriction enzymes *Nhe*I/*Bam*HI to generate pCEP4/BDNF. The pCEP4 vector contains the hygromycin resistance gene, allowing selection of cells which stably integrate the plasmid by virtue of their resistance to hygromycin, and the cloned sequence is driven by the CMV promoter. The BDNF/pCEP4 plasmid was sequenced before transfection to ensure that the cloned BDNF cDNA was full length and in the proper orientation. The pCEP-BDNF construct was transfected into the parental RN33B cells with Lipofectamine® (BRL, Gaithersburg, MD) and clones that stably integrated the construct were selected with 200 μg/ml hygromycin. The maintenance dose is 100 μg/ml hygromycin for proliferation of the cell lines at 33°C. Individual clones were picked with cloning rings and expanded by serial dilution into cells lines as described previously (Whittemore and White, 1993). Similar methods were used to transfect the pCEP4 vector into RN33B cells and the 33V1 (vector-only) cell line was isolated and used as the negative control for all experiments.

2.2. Cell culture of 33BDNF.4 and 33V1 cell lines

At permissive temperature (33°C), 33BDNF.4 and 33V1 cells were grown in Dulbeco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1 vol; D/F media), 10% fetal bovine serum (FBS), the antibiotic G418 (125 μg/ml), and the antibiotic hygromycin (150 μg/ml) as described previously (White et al., 1994). To differentiate 33BDNF.4 and 33V1 cells, 75–90% confluent cells were shifted to 39°C (non-permissive temperature) and the medium was changed to B16 base medium (Brewer and Cotman, 1989) containing 1% (w/v) B16 base, 1% bovine serum albumin (BSA; Boehringer Mannheim), TCM (TCM proprietary serum-free replacement ingredients (CELOX Labs)), and pen-strep. For all in vitro experiments, the 33BDNF.4 cells were differentiated for 3–7 days at 39°C (non-permissive temperature).

2.3. BDNF ELISA

Methods for quantification of mature BDNF protein by enzyme-linked absorbance assay (ELISA) were adapted from procedures described previously (Eaton et al., 1990) and utilized materials and procedures from a BDNF ELISA kit (supplied by Promega, Madison, WI). Recombinant human BDNF supplied in the kit was used to generate a

standard curve of reactivity. Nunc MaxiSorp™ 96-well flat-bottom ELISA plates were coated with 100 μl/well of anti-BDNF monoclonal antibody in carbonate buffer (pH 9.7; 1:1000 dilution) overnight at 4°C. To avoid non-specific binding, 200 μl/well of blocking/sample buffer was incubated for 1 h with the plates. To determine cell numbers in cultures, sister cultures of identically-plated cells were proliferated or differentiated at permissive and non-permissive temperatures, respectively, and counted by Trypan Blue exclusion to determine the BDNF content in the two cell lines. The cells were lysed with sonication, the membranes were removed with high speed centrifugation, and the supernatant was applied to the ELISA plates. To measure BDNF secretion, cells were counted by Trypan Blue exclusion after removal of the media for analysis. After washing the wells, 100 μl/well of tissue-culture media from 33BDNF.4 and 33V1 clones proliferated for 3 days at 33°C (media unchanged for 3 days), or differentiated for 3 days at 39°C (media unchanged for the last 3 days of 7 days of differentiation), and diluted in blocking/sample buffer was kept for 2 h at room temperature on the plates. The plates were then incubated for 2 h with anti-BDNF polyclonal antibody (100 μl/well in blocking/sample buffer; IgY). The plate was then incubated for 1 h with anti-IgY horseradish peroxidase conjugate in blocking/sample buffer (1:2000 dilution), and 100 μl/well of the peroxidase substrate in TMB solution was added for color development. After approximately 10 min the reaction was stopped with 100 μl/well of 1 M phosphoric acid, and the absorbance at 450 nm was recorded. At least three replicates for each cell line at each temperature condition from two independent experiment were used to determine the content and secretion of mature BDNF protein.

2.4. RT-PCR analysis of BDNF mRNA in cell lines

BDNF mRNA expression in the cell lines at permissive and non-permissive temperatures in vitro was analyzed with reverse transcriptase/polymerase chain reaction (RT/PCR) methods (Cejas et al., 1999). First strand cDNA was synthesized using 2 μg of total RNA with the SuperScript II RNAse H reverse transcriptase (RT) (GIBCO, Gaithersburg, MD) enzyme and random priming. Prior to the RT reaction all RNA was DNase-treated to remove any genomic DNA. PCR reactions were performed with BDNF specific primers (5′ primer, 5′-agcctcctctgctctttctgc-3′ and 3′ primer, 5′-cttttgtctatgcccctgcagcctt-3′), which resulted in a 296-base pair (bp) product of the rat BDNF gene.

2.5. Bio-activity assay for BDNF in cell-line conditioned media: DRG cultures

The use of DRG cultures and RT97 neurofilament staining to assay the bio-activity of BDNF to affect neurite outgrowth has been reported (Rosano et al., 1997) and was used here to examine the BDNF bio-activity of conditioned media (CM) from the 33BDNF.4 and 33V1 cell lines used for transplantation. Preparation of embryonic day 15 (E15) DRG explant cultures was has been thoroughly described by Kleitman et al. (1998). E15 Sprague–Dawley embryos were dissected, the spinal ganglia was explanted to wet (rat-tail) collagen-coated Aclar minidishes (three to four DRGs per minidish) in D/F media, 10% FBS, and pen-strep, and cultures were settled for a few hours to attach at 37°C. Then CM from cell lines was used to replace and re-feed explants every other day for 7 days. Equal numbers of 33BDNF.4 and 33V1 cells were grown at a permissive temperature (33°C) as above in T-25 flasks with 6 ml of D/F media, 10% FBS, and pen-strep, with no G418 or Hygro antibiotics present, as a source of CM to feed DRG cultures during the 7 days. After the last feeding, the T-25 flasks were counted by Trypan Blue exclusion to insure that equal numbers of cells were present in the two flasks (about 3×10^6 cells/flask). DRG explants were fixed at 4 or 7 days before fluorescent immunostaining with the anti-neurofilament RT97 antibody.

2.6. Immunohistochemistry

2.6.1. In vitro

2.6.1.1. BDNF. Methods for staining cell cultures for BDNF have been adapted from methods described elsewhere (Eaton and Whittemore, 1996; Eaton et al., 1996). Cell cultures were fixed with modified Zamboni's fixative (Holets et al., 1988) while at 33°C and after 7 days of differentiation at 39°C. The cells were washed with Dulbeco's phosphate buffered saline (DPBS) and treated with 200 μl/well of permeabilization buffer (Triton-X 100 (TX), 0.25% in DPBS) for 30 min. Cells were then incubated with anti-BDNF (rabbit polyclonal; 1:1000 dilution; Chemicon, Temecula, CA) in permeabilization buffer overnight at 4°C. Cells were rinsed and incubated with anti-rabbit IgG Alexa Green fluorescent secondary reporter (Molecular Probes, Eugene, OR) in permeabilization buffer (1:150 dilution) for 1 h. After reactions were completed, slides were cover-slipped using no-fade mounting medium. Photo images were taken from stained eight-well slides of cultures with a Zeiss AxioplanII on TMAX400 professional film and scanned at 200 dpi into TIF format. Images were collected and arranged in Adobe Photoshop.

2.6.1.2. RT97. Methods to identify neurite outgrowth from DRG cultures utilized the RT97 mouse monoclonal antibody directed against the phosphorylated 200 kDa neurofilament protein and developed by J.N. Wood (Kahn et al., 1987). After DRG cultures were treated with CM from both 33BDNF.4 and 33V1 cells for 4 or 7 days, DRG cultures were fixed with modified Zamboni's fixative (Holets et al., 1988) and treated with 1 ml/minidish of permeabilization buffer (Triton-X 100 (TX), 0.4% in DPBS) for 10 min. Cells were then incubated with the RT97 antibody (mouse monoclonal; 1:10 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA) in permeabilization

buffer overnight at 4°C. Cells were rinsed and incubated with anti-mouse IgG Alexa Green fluorescent secondary reporter (Molecular Probes, Eugene, OR) in permeabilization buffer (1:50 dilution) for 1 h. After reactions were completed, slides were cover-slipped using no-fade mounting medium. Photo images were taken from stained DRG cultures with a Zeiss AxioplanII fluorescent microscope on Ektachrome 160T professional film and scanned at 200 dpi into TIF format. Images were collected and arranged in Adobe Photoshop.

2.6.2. In vivo

In a group of animals different than that used to assess sensory behaviors after cell transplants, spinal cords were fixed weekly by transcardial perfusion fixation for survival and immunohistochemical staining 1–7 weeks after CCI and transplantation. The fixative, consisting of 4% paraformaldehyde, 10% picric acid in DPBS, also contained 0.1% glutaraldehyde to localize the various antigens in the grafted cells. After removal from the vertebral column, cords were stored in fix for 12 h at 4°C before the tissue was processed for paraffin embedding. After embedding, cords were thin sectioned (10 μm) and sagittal sections were collected serially to poly-lysine-coated slides. Slides were cleared for 5 min in xylene, re-hydrated and boiled in citrate buffer (pH 7.4) for 15 min at 65°C before beginning the immunostaining procedures. Every second section was stained for bromodeoxyuridine (BrdU) or BDNF and dehydrated, cleared, and mounted in Permamount (Sigma) after antibody staining. The secondary reporter used for BrdU and BDNF localization was the mouse Elite ABC kit (Vector Labs, Inc., Burlingame, CA) with substitution of the DAB substrate with 'VIP' substrate (very intense purple, Vector). Photo images were taken from stained slides of sections with a Zeiss AxioplanII on Ektachrome Elite 400 professional film and scanned at 200 dpi into TIF format. Images were collected and arranged in Adobe Photoshop.

Rats were euthanized for tissue fixation and after the last measure of sensory behaviors by a combination of pentobarbitol overdose and exsanguination. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbitol (12 mg/100 g). Once the appropriate level of anesthesia was reached (i.e. no corneal or withdrawal reflexes), the rat was transcardially perfused with aldehydes. After perfusion, the spinal cord and transplant were removed and histologically processed. All surgical interventions, pre- and post-surgical animal care, and euthanasia were in accordance with the Laboratory Animal Welfare Act, Guide for the Care and Use of Laboratory Animals (NIH, DHEW Pub. No. 78-23, Revised, 1978) and guidelines provided by the Animal Care and Use Committee of the University of Miami, Miami, FL, which conform to the guidelines on the study of pain in awake animals established by the International Association for the Study of Pain (Zimmermann, 1983).

2.6.2.1. BrdU. Cells used for transplants and antibody staining were treated with 10 μM BrdU during proliferation for 3 days before transplantation and grafts were stained for BrdU post-transplantation as described previously (Eaton et al., 1999b). The nucleic acid BrdU, followed by an anti-BrdU antibody (Becton Dickinson, Mountain View, CA), has been commonly used (Yong and Kim, 1987) to mark cell nuclei and was used here to mark the surviving cells transplanted (Seiler and Aramant, 1995) in the subarachnoid space of the lumbar spinal cord. Briefly, sections were re-hydrated in DPBS buffer, followed by 2 N HCl for 30 min, and rinsed with DPBS. They were then placed in borate buffer for 15 min (borate buffer: borax, 0.95 g/50 ml water and boric acid, 0.625 g/50 ml water (1:1)) and rinsed twice with DPBS. Next, they were permeabilized with 0.02% TX/DPBS for 5 min, followed by a DPBS rinse, and incubated with the anti-BrdU antibody (1:20 dilution/DPBS) overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and 'VIP' substrate (Vector).

2.6.2.2. BDNF. The methods used to identify BDNF immunoreactivity in grafted cells is a modification of methods previously described (Eaton and Whittemore, 1996; Eaton et al., 1996). After removal from the vertebral column, cords were stored in fix for 12 h at 4°C before paraffin embedding and thin sectioning. The anti-BDNF antibody (1:500/DPBS; Chemicon) was incubated with TX-permeabilized (0.2%/DPBS) sections overnight at 4°C, followed by an anti-rabbit peroxidase ABC reporter (Vector) and 'VIP' substrate (Vector).

2.7. Transplantation of 33BDNF cells

Transplantation of neuronal rat cell lines into the subarachnoid space of the lumbar spinal cord after CCI has been described previously (Eaton et al., 1999b). The 33BDNF.4 and 33V1 cell lines were grown at a permissive temperature (33°C), as described above (Eaton et al., 1999b). Both the 33BDNF.4 and 33V1 cells were proliferated at 33°C to near confluence before use in grafting. Immediately before transplantation, these proliferated cells were gently dissociated from six-well culture plates with sterile 0.5 mM EDTA/DPBS, pelleted by centrifugation, the viability and cell numbers assessed by Trypan Blue exclusion, and the cells suspended in 10° μl of Ca^{2+}–Mg^{2+}-free Hank's buffered saline solution (CMF-HBSS). An aliquot of one million cells was prepared immediately prior to each transplant to assure near 100% viability at the beginning of the experiment; grafting was within 30 min of cell preparation. Following a partial laminectomy and a small puncture of the dura, viable cells (10^6) were injected into the subarachnoid space of the lumbar dorsal spinal cord by a dorsal/caudal entry into the dural puncture a few millimeters along the caudal surface with a small length of polyethylene (PE-10) tubing containing the cells at spinal segment L1 at 1 week after the CCI in animals that demonstrated a vigorous

nociceptive effect of the nerve injury. For both CCI and transplantation, animals were anesthetized with a mixture of ketamine, xylazine, and acepromazine (0.65 ml/kg). Animals were allowed to recover at 37°C for 12 h, after which time they were returned to the animal care facility, and housed one per cage with rat chow and water ad lib on a 12 h light/dark cycle.

2.8. Chronic constriction injury (CCI)

The surgery to produce CCI was first described by Bennett and Xie (1988). This model of injury has been used to test the ability of cell transplants to relieve the pain-related behaviors (Eaton et al., 1997, 1999b), and was subsequently used before transplantation of the similarly-derived and bio-engineered 33BDNF.4 and 33V1 cell lines. Under ketamine/xylazine anesthesia, the right common sciatic nerve was exposed at the level of the middle thigh by blunt dissection through the biceps femoris. Proximal to the nerve's trifurcation, 5–7 mm of nerve was freed of adhering tissue and four ligatures (4.0 chromic gut) were tied loosely around it with about 1 mm spacing. Care was taken to tie the ligatures so that the diameter of the nerve was barely constricted. The incision was closed in layers and the entire surgery was repeated, minus the ligatures, on the left side to create a sham-operated nerve.

2.9. Behavioral testing

One week before CCI and transplants, animals were acclimated and trained for 3 days on all behavioral tests, followed by a baseline measure of the tests described below 1 week before CCI. One week following CCI and every week thereafter animals were re-tested. Surgery and sensory testing were done in separate locations by different experimenters on animals with unmarked cage cards. All animals, transplanted and non-transplanted, received near-identical anesthesia and midline incisions near the transplant area at the same time point in the experiment, 1 week after CCI. Only animals that demonstrated a vigorous hypersensitivity to tactile and thermal stimuli 1 week after CCI were transplanted 1 day later. Animals to be transplanted were then injected with either the control 33V1 or the 33BDNF.4 cells. A third group of animals received CCI but no transplants and served as the CCI-alone control group. A fourth group of animals received neither CCI nor transplants and served as the naive control animals. A fifth group of animals received transplants but not CCI and served as transplant-only controls. The behavioral data resulting from these animals did not demonstrate results different from the non-transplanted, non-injured animals, and hence were not included in the data figures. Behavioral testing was repeated for all animals once a week for 8 weeks following CCI and transplants. All animals used for behavioral testing were sacrificed after 8 weeks of testing. Another group of animals used to examine chronic survival of cells after CCI and transplantation were sacrificed weekly and were not included with those used for behavioral testing.

2.9.1. Cold allodynia

Methods for testing the response to cold stimuli have been described elsewhere (Bennett and Xie, 1988). Each rat was placed under a transparent plastic cover on a cold copper plate (4 ± 1°C). After 5 min of adaptation, the number of hindpaw lifts for both right (ligated) and left (sham-operated) paws during a 20 min interval were counted. The total duration of hindpaw lifts for each paw over the 20 min interval was recorded. For each session, for each animal, the score of the sham-operated paw was subtracted from the ligated paw. From these values, a mean difference score for each session was calculated for each group of animals and used to determine the effects of the different treatments. Responses were measured once weekly before and after surgery and transplantation.

2.9.2. Thermal hyperalgesia

Methods for testing thermal hyperalgesia with a Hargreaves device have been described elsewhere (Hargreaves et al., 1988). Animals were placed in a clear plexiglass box on an elevated plexiglass floor and allowed to acclimate for approximately 5 min. A constant intensity radiant heat source was aimed at the midplantar area of the ligated and sham-operated hindpaws. The time, in seconds, from initial heat source activation until paw withdrawal was recorded. Five minutes were allowed between stimulations. Five latency measurements for each paw were recorded at weekly sessions before and after CCI and after transplantation. For each of the five latency measurements, the score of the sham-operated paw was subtracted from the ligated paw. From these values, a mean difference score for each session was calculated for each experimental animal and used to determine the effects of the different treatments.

2.9.3. Mechanical allodynia

Mechanical allodynia, the occurrence of foot withdrawal in response to normally innocuous mechanical stimuli, was tested using a graded series of von Frey hairs (Chaplan et al., 1994). Animals were placed in a plexiglass box with an elevated mesh floor. After the animal was acclimated for 5 min, calibrated von Frey hairs with ranges from 0.41 to 8.5 g were applied perpendicular to the midplantar area of the ligated and sham-operated hindpaw and depressed slowly until bent. The value, in grams, for the minimal initial hindpaw withdrawal was recorded for each of five trials. A single trial of stimuli consisted of five applications of a von Frey filament within a 10 s period to insure that the response was constant. Each session consisted of five trials repeated at 3 min intervals on each hindpaw. This minimum value for the initial response for the sham-operated paw was subtracted from the score for the ligated paw and all five replicate scores were averaged. From these values, a difference

score was calculated and used to determine the effects of the different treatments.

2.9.4. Mechanical hyperalgesia

Mechanical pain thresholds to noxious stimulation (paw pinch) after cell transplants (Sagen et al., 1987) and CCI (Bennett and Xie, 1988) were determined with a modified version of the Randall–Selitto method (Randall and Selitto, 1957), using a paw pinch analgesymeter (Stoelting, Wood Dale, IL). A conical stylus with a hemispherical tip (1.2 mm radius) was placed on the middle of the hindpaw dorsal surface. The animal was restrained gently between cupped hands and calibrated pressure of gradually increasing intensity (16 g/s) was applied until the rat withdrew the hindpaw. The hindpaws were tested alternately at 3–4 min intervals. Five measurements were taken for each side and averaged, and the difference score was computed by subtracting the average of the control side from the average of the ligated side. From these values, a mean difference score for each session was calculated for each experimental animal and used to determine the effects of the different treatments.

2.10. Chemicals

Primary antiserum used for in vitro and in vivo immunohistochemistry against the indicated antigens was obtained from the following sources: 5-bromo-2′-deoxyuridine (BrdU), Becton Dickinson (Mountain View, CA); BDNF, Chemicon (Temecula, CA); and RT97, mouse monoclonal antibody directed against neurofilament proteins, Developmental Studies Hybridoma Bank (Iowa City, IA). BDNF ELISA kit materials were supplied by Promega (Madison, WI). For tissue culture, CNS medium (Kawamoto and Barrett, 1986) and Geneticin (G418) were obtained from GIBCO (Grand Island, NY); hygromycin and all other powdered media, attachment factors and chemicals were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA; fraction V, protease-free) was obtained from Boehringer Mannheim (Indianapolis, IN), fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT), and tissue culture plasticware was obtained from various commercial suppliers. Restriction enzymes and DNA modifying enzymes were purchased from Pharmacia (Piscataway, NJ). The pCEP4 expression vector was obtained from Invitrogen (San Diego, CA).

2.11. Statistical analysis

The statistical significance of all quantitative data was determined with a multivariate analysis of variance (MANOVA). Comparisons of differences between individual means were tested using the Tukey honest significant differences (HSD) method or the unequal *N* least significant difference (LSD) test. All of the analyses were performed with a commercially available software package (Statistica, Statsoft, 1990). *P*-values less than 0.05 (*) or less than 0.001 (**) were considered statistically significant.

3. Results

3.1. BDNF immunoreactivity for protein and RT-PCR for BDNF mRNA in cell lines in vitro

After isolation of the clonal cell line 33BDNF.4, transfected with the rat BDNF gene, these cells were examined in vitro for expression of the mature BDNF protein and mRNA at proliferation and differentiation conditions (33 and 39°C, respectively). Immunoreactivity for BDNF protein and RT-PCR for mRNA in the bio-engineered cell line was compared to the control cell line, 33V1, which was transfected with the pCEP4 only. The 33BDNF.4 and 33V1 cell lines were cultured in vitro (Fig. 1) and most 33BDNF.4 cells stained for BDNF immunoreactivity after proliferation at a permissive temperature (33°C) (Fig. 1A), while all 33BDNF.4 cells contained intense BDNF immunoreactivity after differentiation for 7 days at a non-permissive temperature (39°C) (Fig. 1C). Similar cultures of cells with the pCEP4 vector only, the 33V1 cells, contained only barely detectable BDNF immunoreactivity after proliferation (Fig. 1B) or differentiation for 7 days (Fig. 1D).

Similarly, BDNF mRNA in cultured cells was examined with RT-PCR methods in 33V1 and 33BDNF.4 cell lines at permissive (proliferation) and non-permissive (differentiation) temperatures in vitro (Fig. 2). The 33BDNF.4 cell line contained significant BDNF mRNA (lane 4), while the cells were proliferating at a permissive temperature. After 7 days of differentiation at a non-permissive temperature, BDNF mRNA continued to be expressed in 33BDNF.4 cells (lane 5). Low levels of BDNF mRNA were present in the 33V1 control cells, both during proliferation (lane 2), and after 7 days of differentiation (lane 3) by RT-PCR methods. A 296 bp product, identified as BDNF by sequencing, resulted.

3.2. ELISA for BDNF secretion in conditioned media from cell lines in vitro

The immortalized 33BDNF.4 and 33V1 cell lines were cultured in vitro at permissive and non-permissive temperatures, when the cells were proliferating and differentiating, respectively, and the BDNF content and secretion of BDNF from the cells into the medium were examined with ELISA methods (Fig. 3). The very low levels of BDNF mRNA and protein immunoreactivity expressed by the control 33V1 cells was reflected in the ELISA results for the cell line. The total BDNF contained in 33V1 cells was 1.28 ± 0.23 ng/10^6 cells at 33°C and 0.95 ± 0.08 ng/10^6 cells at 39°C. In contrast, the total mature BDNF contained by the 33BDNF.4 cells was 15.08 ± 0.93 ng/10^6 cells at 33°C and 8.07 ± 0.42 ng/10^6 cells at 39°C. The ability to measure BDNF in the media conditioned by the cells reflected their ability to constitutively secrete the BDNF protein. The rate of BDNF secreted by the control 33V1 cells was 0.58 ± 0.02 ng/10^6 cells per day at 33°C and 0.16 ± 0.16 ng/10^6 cells per day at 39°C. In contrast, the rate of BDNF secretion

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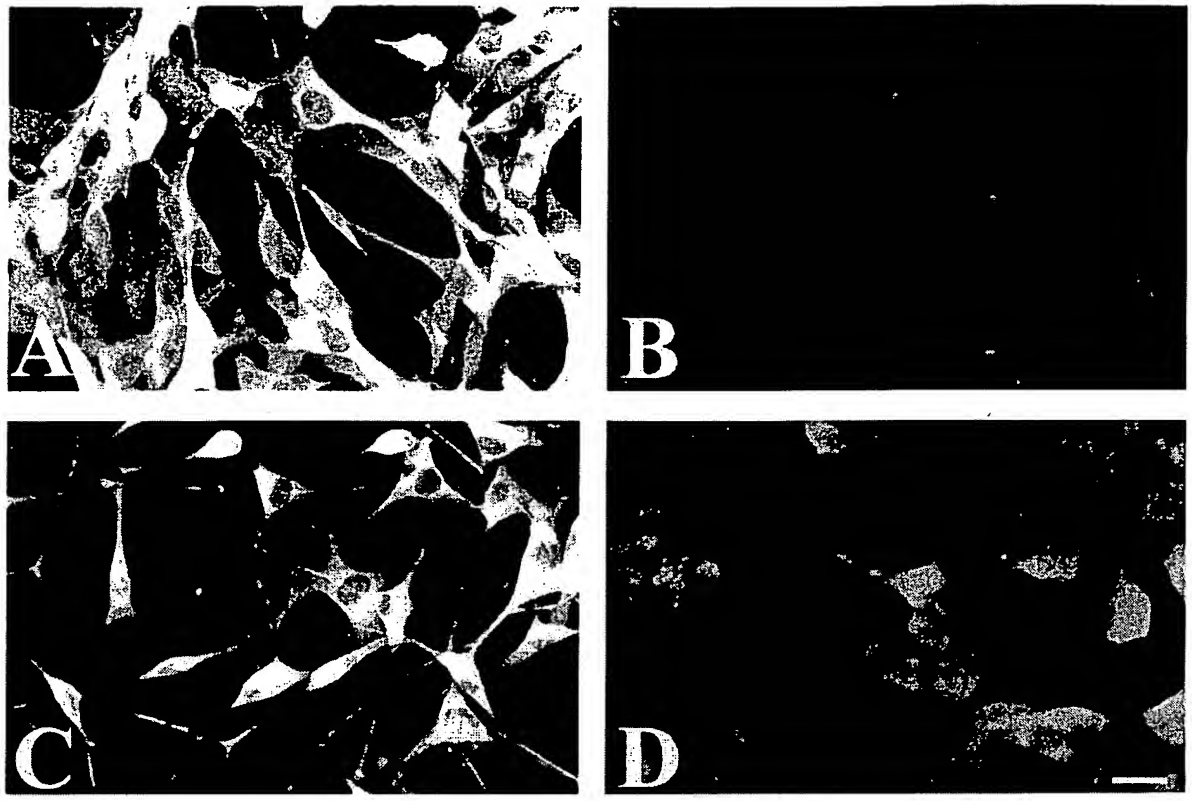

Fig. 1. BDNF immunoreactivity at 33° and 39°C in vitro. After proliferation at 33°C in vitro, the 33BDNF.4 (A) and 33V1 (B) cells were stained with an antibody for BDNF. After 7 days of differentiation at 39°C in vitro, the 33BDNF.4 cells remain BDNF (+) (C), while 33V1 cells continue to be BDNF (−) (D). Magnification: bar, 150 μm.

by the 33BDNF.4 cells was 26.65 ± 1.80 ng/10^6 cells per day at 33°C and 2.52 ± 0.28 ng/10^6 cells per day at 39°C.

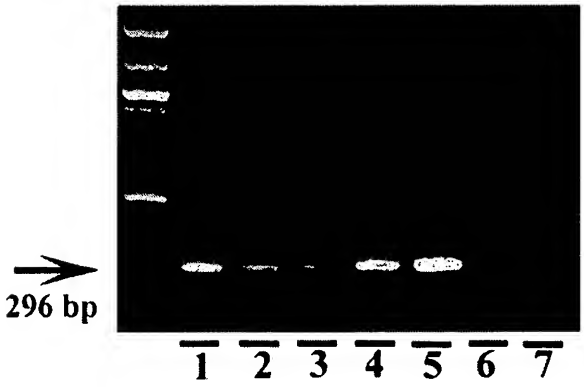

Fig. 2. RT-PCR for BDNF mRNA in cells at 33° and 39°C in vitro. Total RNA was isolated and 2 μg was reverse transcribed followed by PCR with BDNF specific primers. The resulting product was 296 bp in length. Sequencing of the PCR products and agarose gel electrophoresis revealed significant levels of BDNF mRNA in 33BDNF.4 cells (at 33°C) (lane 4) and after 7 days at 39°C (lane 5). Low levels of BDNF mRNA were expressed by 33V1 cells at 33°C (lane 2) and after 7 days at 39°C (lane 3). RNAse-treated RNA from 33BDNF.4 cells grown at 33°C (lane 6) and 39°C (lane 7) were negative for BDNF mRNA. Brain stem RNA served as the positive control (lane 1). Molecular weight markers are in the first unmarked lane.

The secretion rate for 33BDNF.4 cells at 33°C reflects the amount of BDNF secreted by these cell grafts soon after grafting into the subarachnoid space.

3.3. Bio-active BDNF in CM from cell lines in vitro: DRG cultures

Although retinal ganglion survival assay has been used to examine the bio-activity of BDNF, use of DRG explant culture and neurite outgrowth is a more direct method to assay bio-activity of BDNF, and was used here to compare the CM from the 33BDNF.4 and 33V1 cell lines grown at permissive temperatures in vitro. The effects on neurite outgrowth from E15 DRG explant cultures after treatment with the CM from the two cell lines is illustrated in Fig. 4. Only minimal or barely detectable neurite outgrowth is visible after 4 days of treatment of DRG explant cultures with CM from the negative control cell line, 33V1 (Fig. 4B), while after 4 days of exposure to CM from the 33BDNF.4 cell line, neurite outgrowth is dramatically increased (Fig. 4A). After 7 days of treatment with CM from the two cell lines, DRG explants show little neurite outgrowth when 33V1 cells are used as a CM source (Fig. 4D). This is comparable to treatment of the DRGs with non-conditioned media (data not shown). However, after 7 days of treatment

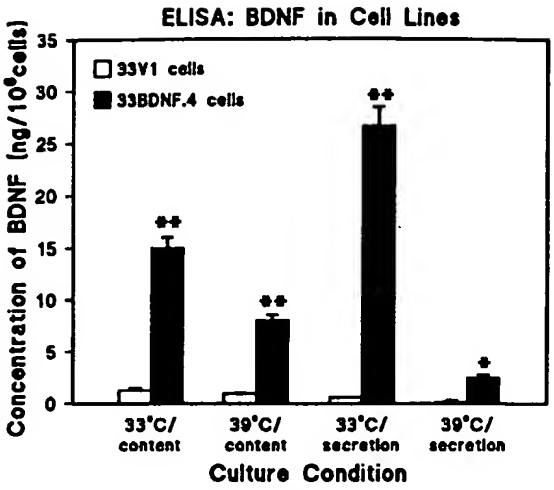

Fig. 3. ELISA for BDNF content and secretion in cells at 33° and 39°C in vitro. ELISA methods were used to examine the production and rate of secretion of BDNF from the 33BDNF.4 and negative control 33V1 cell lines in vitro. Media was left on the cells over a 3 day time period to assay for secretion into the media. The data reported are the mean ± SEM from at least three replicates of each cell line at each temperature from two independent experiments to determine the total content/10^6 cells and the secretion/10^6 cells per day. Post-hoc analysis of the data indicated that the 33BDNF.4 cells were synthesizing and secreting significant amounts of mature BDNF compared to the negative 33V1 control cells. Asterisks indicate that BDNF production for 33BDNF.4 cells differed significantly from the 33V1 cells at each condition; $*P < 0.05$, $**P < 0.001$.

with CM from 33BDNF.4 cells, neurite outgrowth is dramatically increased (Fig. 4C).

3.4. Chronic survival and BDNF synthesis in grafted cells after transplantation

Chronic survival of transplants was evaluated in 33BDNF.4 and 33V1 cells with anti-BrdU immunohistochemistry at weekly intervals after transplant placement and in all the animals used for sensory data at the end of the experiment (7 weeks after grafting). All animals used for sensory testing after nerve injury and cell grafts contained numerous grafted cells, both in animals that received the 33BDNF.4 cells and control 33V1 cells. Examples of transplant survival of both cell line grafts 7 weeks after transplant are seen in Fig. 5. Many cell nuclei positive for BrdU immunoreactivity in both cell types were found throughout the 7 weeks after transplant (data not shown). Both types of grafted cells attached to the spinal pial surface near the transplant site and were found perispinally several segments rostral and caudal to the L1–2 injection site at 7 weeks after grafting (Fig. 5A,B). However, while many BrdU immunoreactive cells were found after transplant of 33V1 cells (Fig. 5B), they did not stain for BDNF at any time through 7 weeks after transplant (Fig. 5D). The 33BDNF.4 graft site always contained many BrdU immunoreactive cells on the pial surface (Fig. 5A), most of which were BDNF (+) at the end of behavioral testing, 7 weeks after lumbar transplant (Fig. 5C).

3.5. Thermal hyperalgesia after transplants

The measure of sensitivity to noxious heat in animals after nerve injury and cell transplants is shown in Fig. 6. In control animals without surgery or transplants, no difference was observed between operated and unoperated hindlimbs in the latency of withdrawal over 8 weeks. The range of latency scores was 6.3–16.9 s in the right hindpaw and 6.1–17.6 s in the left hindpaw at 2 weeks in control animals. After CCI, a vigorous hypersensitivity to heat was observed in the ligated paw 1 week after CCI that reached maximal at 1–2 weeks, and did not recover by 8 weeks. At 2 weeks after CCI, the range of latency scores was 5.0–10.9 s in the ligated (right) hindpaw and 6.5–18.2 s in the non-ligated (left) hindpaw. A similar sensitivity was seen in the ligated paw before transplantation of 33BDNF.4 cells at 1 week after CCI. However, 2 weeks after transplantation of 33BDNF.4 cells (3 weeks after CCI), the sensitivity in the ligated hindpaw completely disappeared. The range of latencies was 6.8–16.8 s in the ligated hindpaw and 7.0–16.5 s in the non-ligated hindpaw at 2 weeks after transplant of 33BDNF.4 cells. After transplant of 33V1 cells, the hypersensitivity observed after CCI in the ligated hindpaw remained for 8 weeks after the CCI. The range of latency scores at 1 week after transplant of 33V1 cells was 4.1–11.1 s in the ligated hindpaw and 6.8–18.1 s in the non-ligated hindpaw.

3.6. Tactile hyperalgesia after transplants

The effects of transplants of 33BDNF.4 and 33V1 cells on tactile hyperalgesia, or paw pinch, are shown in Fig. 7. Naive animals demonstrated a similar sensitivity in each hindpaw, with the range of latency scores being 8.0–13.0 in the left paw and 7.0–15.0 in the right paw. One week after CCI, the maximum response to paw pinch was seen in the ligated hindpaw and this level of hypersensitivity continued throughout the 8 weeks after CCI. The range of raw latency scores at 1 week after CCI was 4.0–7.0 in the ligated hindpaw and 8.0–15.0 in the non-ligated hindpaw. The 33BDNF.4 cells, placed 1 week after CCI, potently reversed the nociception in the ligated hindpaw 2 weeks after transplantation, with the hindpaw developing a hyposensitivity to paw pinch. At 2 weeks after transplantation the range of raw latency scores was 8.0–14.0 in the ligated hindpaw and 7.0–15.0 in the non-ligated hindpaw. In contrast, after transplantation of 33V1 cells, the hypersensitivity to paw pinch was not reversed throughout the 8 week course of the experiment. At 2 weeks after transplant, the raw latency scores were 3.0–7.0 in the ligated hindpaw and 9.0–15.0 in the non-ligated hindpaw.

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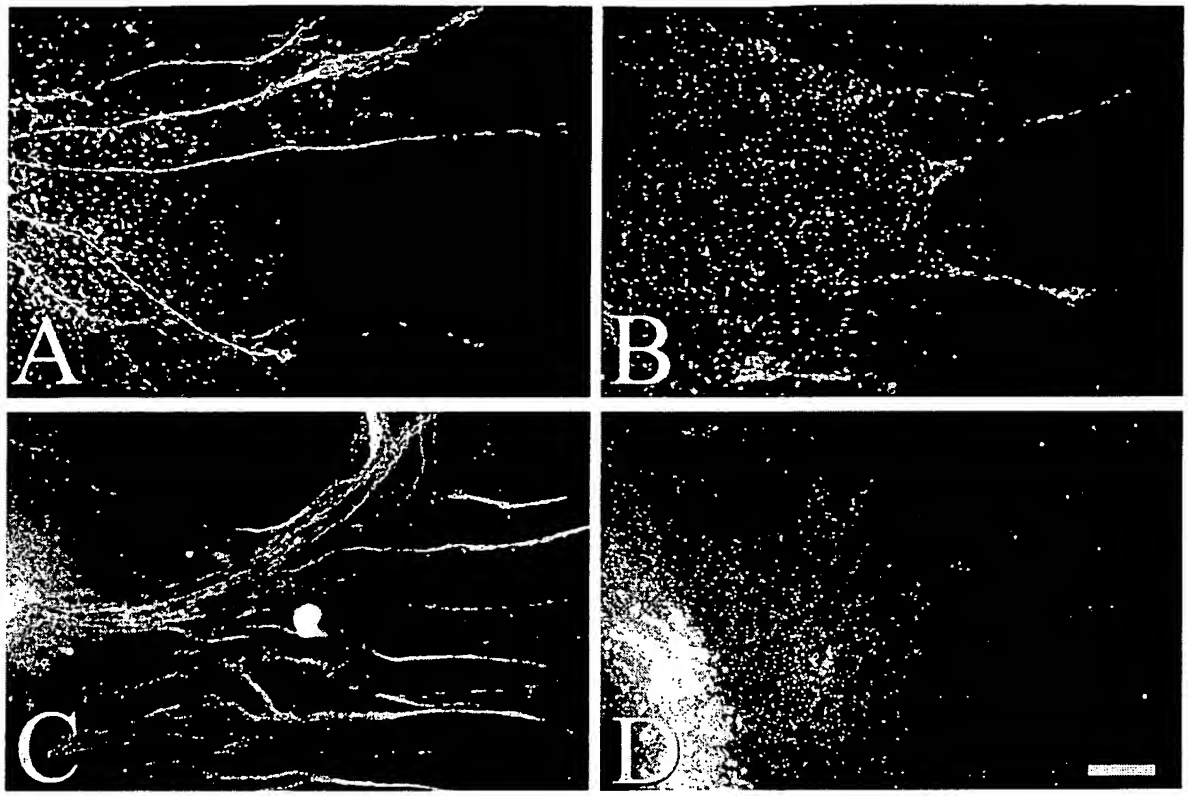

Fig. 4. DRG explant culture assay for BDNF bio-activity from cell lines in vitro. The conditioned media (CM) from equal numbers of 33BDNF.4 and 33V1 cells grown at a permissive temperature (33°C) was fed to E15 DRG explant cultures over 7 days. At 4 (A,B) and 7 days (C,D) DRG cultures were fixed and stained with RT97 anti-neurofilament antibody to identify the effects of CM on neurite outgrowth. The CM from 33BDNF.4 caused extensive neurite extension at both 4 (A) and 7 days (C) of treatment, while CM from the 33V1 had no effect at either 4 (B) or 7 days (D), even compared to DRGs grown in non-conditioned media (data not shown). Magnification: bar, 100 μm.

3.7. Tactile allodynia after transplants

The effects of transplants of the 33BDNF.4 and 33V1 cells on mechanical allodynia after CCI are shown in Fig. 8. All animals were examined 1 week before CCI and for 8 weeks after CCI for foot withdrawal in response to stimulation with a graded series of von Frey hairs as described in Section 2. A significant response appeared in the ligated hindpaw 1 week after CCI alone that did not resolve during the 8 weeks after surgery. The near maximum nociceptive effect appeared in the ligated paw versus the non-ligated paw at 1 week following CCI, with the range of raw latency scores being 0.025–0.675 g in the ligated paw and 1.22–3.55 g in the non-ligated paw. At the same time period, the scores for the uninjured control animals varied from 1.20 to 3.60 g in either paw. However, 1 week after 33BDNF.4 cells were transplanted near the lumbar spinal cord after CCI, no significant mechanical sensitivity was observed. At this time period, the range of raw latency scores was 0.49–3.60 g in the ligated paw and 3.90–4.08 g in the non-ligated paw. Transplants of the 33V1 cells had no significant effect on the induction of allodynia by CCI. At this same time period, 1 week after transplants of 33V1 cells and 2 weeks after CCI, the range of raw latency scores was 0.030–0.69 g in the ligated paw and 1.20–3.60 g in the non-ligated paw.

3.8. Cold allodynia after transplants

A measure of cold allodynia using hindpaw withdrawal to a cold plate (Fig. 9) demonstrated a vigorous hypersensitivity 1 week after CCI that continually increased through 8 weeks after CCI, and never returned to baseline. At 2 weeks after CCI, the range of latency scores was 10–36 hindpaw lifts with 12.5–80.1 s duration in the ligated hindpaw. Both the number of hindpaw lifts (Fig. 9A) and the total duration of hindpaw withdrawal (Fig. 9B) were significantly reduced after transplantation of 33BDNF.4 cells near the lumbar spinal cord, with the reduction of sensitivity beginning 1 week after transplantation of 33BDNF.4 cells (2 weeks after CCI). The range of raw latency scores at this time point was 2–8 hindpaw lifts in the ligated paw, with no lifts in the non-ligated paw. At the same time point after transplant of 33BDNF.4 cells, the range of duration over 20 min in the ligated hindpaw was 2.5–10.0 s. No cold plate sensitivity was observed in uninjured animals, transplanted uninjured animals, or on the sham-operated hindpaw (data not shown) of any animal in any test group. When 33V1

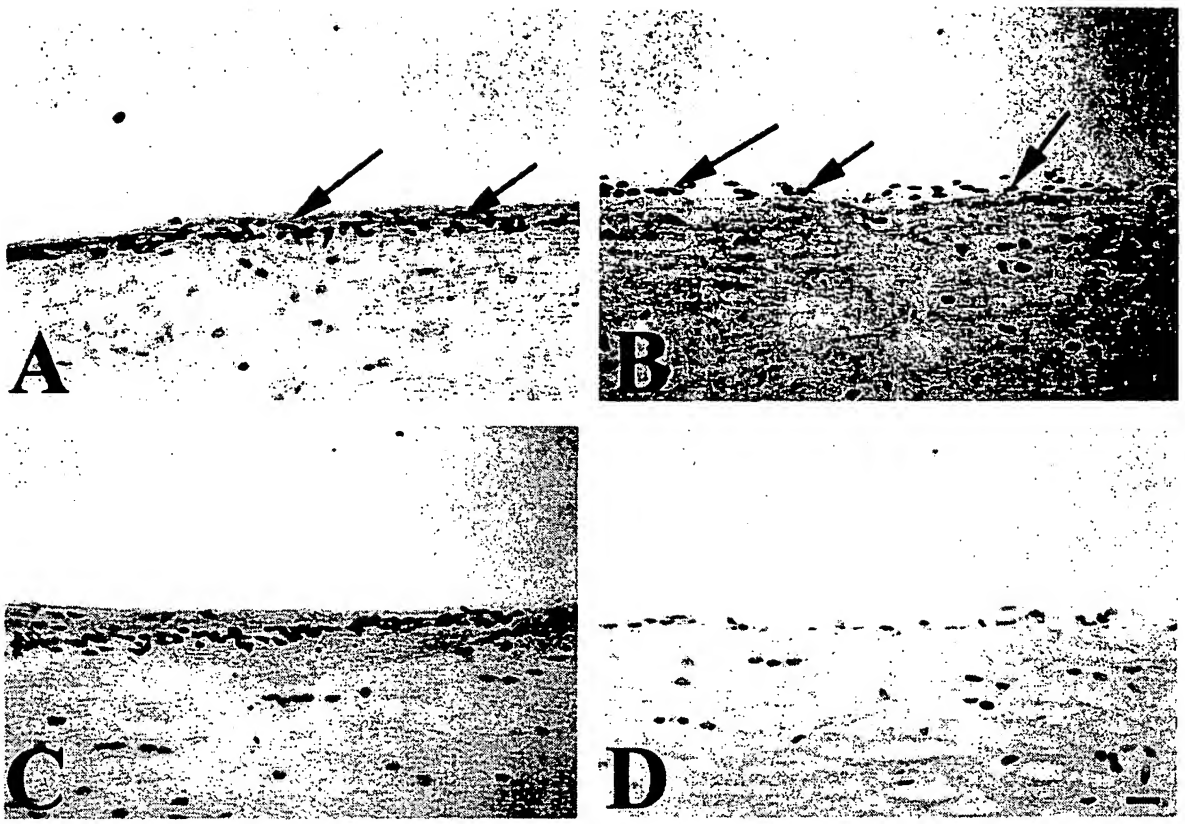

Fig. 5. Transplanted cell lines after nerve injury. Both 33BDNF.4 (A,C) and 33V1 (B,D) cells were transplanted 1 week after CCI in the lumbar subarachnoid space and examined 7 weeks after transplant. Cells were pre-incubated before transplant with BrdU to identify surviving cells in the grafts (A,B; arrows). Sections were immunohistochemically stained for BrdU (A,B) and BDNF (C,D). Only 33BDNF.4 grafts contained surviving cells that were both BrdU (+) and BDNF (+); 33V1 grafts had no BDNF immunoreactive cells, even though many BrdU (+) 33V1 cells survived for >7 weeks after transplant and nerve injury. Magnification: bar, 100 μm.

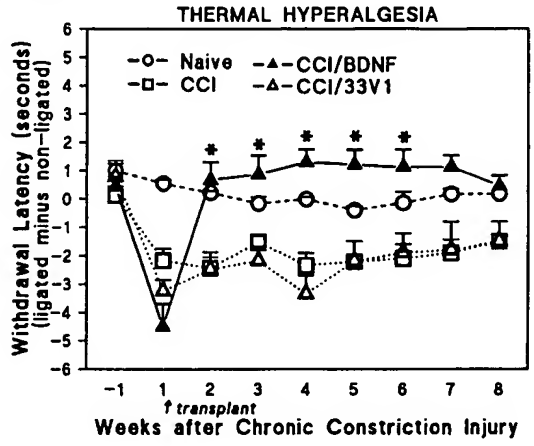

Fig. 6. Thermal hyperalgesia after CCI and transplants. Animals were either left unoperated (○), given the CCI (□), or transplanted with 33BDNF.4 (▲) or 33V1 cells (△) 1 week following the CCI and 1 day following behavioral testing. Animals were tested for hindpaw withdrawal once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated thermal hyperalgesia 1 week after CCI were transplanted. The data reported are the mean ± SEM of the difference values for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate the 33BDNF.4 transplants that differed significantly from the 33V1 cell transplant condition at each time point; $P < 0.05$.

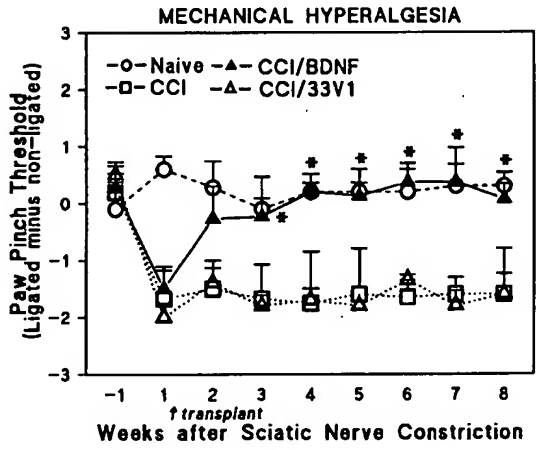

Fig. 7. Tactile hyperalgesia after CCI and transplants. Animals were either left unoperated (○), given the CCI (□), or transplanted with 33BDNF.4 (▲) or 33V1 cells (△) 1 week following the CCI and 1 day following behavioral testing. Animals were tested for hindpaw withdrawal in a paw pinch device once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated tactile hyperalgesia 1 week after CCI were transplanted. The data reported are the mean ± SEM of the difference values for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate the 33BDNF.4 transplants that differed significantly from the 33V1 cell transplant condition at each time point; $P < 0.05$.

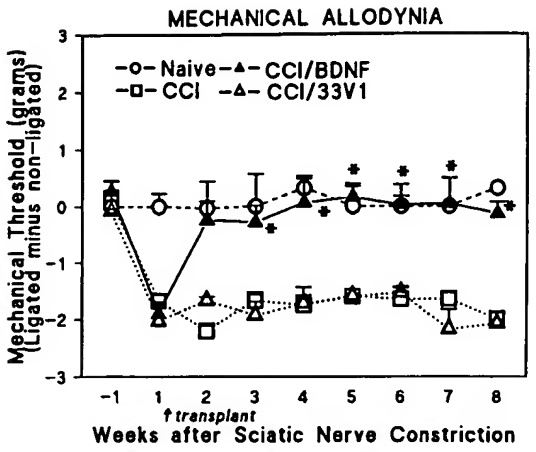

Fig. 8. Tactile allodynia after CCI and transplants. Adult female rats were either left unoperated (O), underwent CCI (□), or transplanted with 33BDNF.4 (▲) or 33V1 cells (△) 1 week following CCI and 1 day following behavioral testing. Animals were tested for hindpaw withdrawal to a graded series of von Frey hairs once every week for 1 week before and 8 weeks following CCI and before and after transplants. Only animals that demonstrated tactile allodynia 1 week after CCI were transplanted. The data reported are the mean ± SEM of the difference scores for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate the 33BDNF.4 transplants that differed significantly from the 33V1 cell transplant condition at each time point; $P < 0.05$.

cells were transplanted after CCI, both the number and duration of hindpaw withdrawals were similar to CCI alone. The range of latency scores was 13–40 hindpaw lifts with 8.8–136.9 s duration over 20 min in the ligated hindpaw.

4. Discussion

The potential site of action of BDNF in the modulation of nociception is the dorsal horn. Various models of nerve injury that produce chronic pain, such as sciatic nerve transection or crush (Cho et al., 1998; Li et al., 1999), increase endogenous BDNF and mRNA transcripts (Enfors et al., 1993) in the DRG (Enfors et al., 1990, 1993). BDNF is also increased in the DRG (Cho et al., 1997b) and dorsal horn (Cho et al., 1997a) following peripheral inflammation. The likely source of endogenous BDNF production is the afferent fibers (Acheson et al., 1995; Zhou and Rush, 1996), probably small caliber C fibers projecting to the dorsal horn (Zhou and Rush, 1996; Cho et al., 1997a; Conner et al., 1997). BDNF is anterogradely transported by the DRG neurons to their target sites (Conner et al., 1997), and this process is accelerated by noxious, peripheral manipulation (Li et al., 1999; Millan, 1999). The original notion that neurotrophins should be grouped by structural homology has been altered with studies that demonstrate that BDNF and other neurotrophins may function differently in different locations and their modes of action are likely heterogeneous (Conner et al., 1998; Fawcett et al., 1998). Another line of evidence for a role for BDNF in chronic pain is the expression of the BDNF receptor trkB (Klein et al., 1991), and its up-regulation in the cord after spinal injury (Frisen et al., 1992) and the distal sciatic nerve after axotomy (Funakoshi et al., 1993). In contrast, in various human peripheral neuropathies (Sobue et al., 1998) that involve inflammatory cell invasions, trkB mRNA levels are diminished.

Assigning only a anti-nociceptive function for endogenous BDNF in neuropathy is difficult. Neurotrophic factors such as BDNF and NT-3 play a significant role in the development, and perhaps maintenance, of the sensory neurons responsive to temperature and tactile pain, as evidenced in neurotrophin knockout mice (Klein, 1994). Both motorneurons and sensory neurons express trkB (Schectersom and

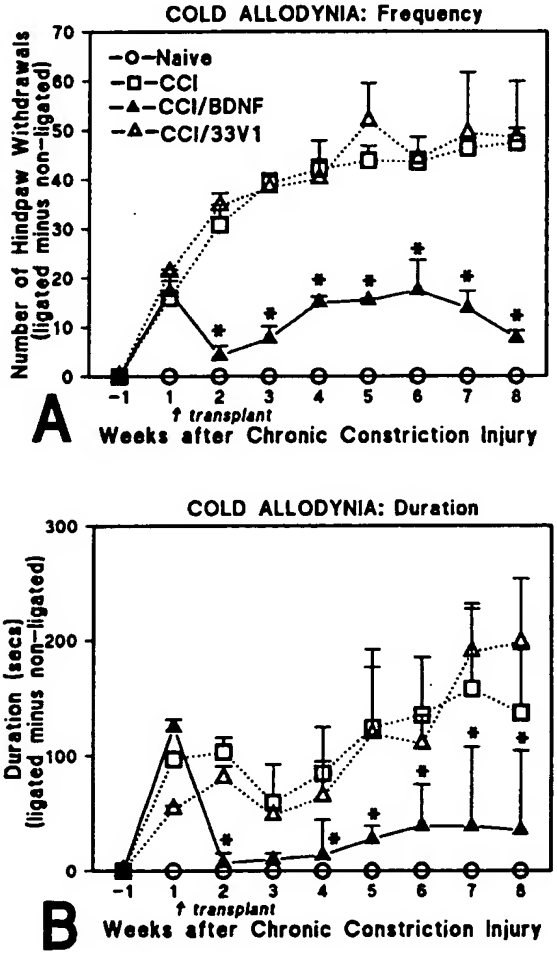

Fig. 9. Cold allodynia after CCI and transplants. The number of hindpaw withdrawals (A) or the total duration of hindpaw lifting (B) in response to a cold plate over 20 min were counted once every week for 1 week before and 8 weeks following CCI and before and after transplants. Animals were either left unoperated (O), underwent CCI (□), or transplanted with 33BDNF.4 (▲) or 33V1 cells (△) 1 week following the CCI and 1 day following behavioral testing. Only animals that demonstrated cold allodynia 1 week after CCI were transplanted. The data reported are the mean ± SEM of the difference values for ligated paw minus the sham-operated paw of 14 animals in each group. Asterisks (*) indicate the 33BDNF.4 transplants that differed significantly from the 33V1 cell transplant condition at each time point; $P < 0.05$.

Bothwell, 1992; Koliatsos et al., 1993; McMahon et al., 1994). Since BDNF is localized to dense core vesicles in terminals in the dorsal horn (Michael et al., 1997), it could be released with synaptic activation of afferents (Griesbeck et al., 1999) and modulate transmission of sensory stimuli. In the peripheral inflammation model, endogenous BDNF release may play a very different role in central sensitization, with increased NMDA receptor-mediated excitability to nociceptive inputs in spinal neurons (Thompson et al., 1999) and induction of c-fos (Kerr et al., 1999) after treatment with exogenous BDNF. Similar regulation of excitatory synaptic strengths by exogenous BDNF has been seen in cortical circuits (Rutherford et al., 1998). This is in contrast to nerve injury where BDNF is able to reduce the $GABA_A$ receptor-mediated conductances in axotomized cutaneous afferents (Oyelese et al., 1997), suggesting that BDNF may facilitate nociceptive input from those terminals into the dorsal horn after nerve injury.

There is significant evidence that exogenous BDNF infusion near the spinal cord provides potent anti-nociception (Frank et al., 1997) by increasing the synthesis and release of serotonin (5HT) in descending raphe serotonergic/peptidergic neurons providing inhibitory modulation of the dorsal horn (Martin-Iverson et al., 1994). Recently, exogenous BDNF was seen to have a direct positive effect on both uninjured (Siuciak et al., 1994) and injured (Xu et al., 1995) serotonergic neurons in the brainstem, increasing 5HT synthesis and promoting regeneration of 5HT axons, respectively. Other peptides such as somatostatin and neuropeptide Y, located in the spinal dorsal horn (Yin, 1995; Munglani et al., 1996; Mark et al., 1997), are up-regulated by BDNF in vitro (Nawa et al., 1993) and in vivo (Croll et al., 1994; Nawa et al., 1994). Both these peptides are regulated in the dorsal horn after various spinal or nerve injuries (Wakisaka et al., 1992; Zhang et al., 1994b), and are believed to play a role in anti-nociception (Duggan et al., 1991; Hua et al., 1991; Chapman and Dickenson, 1992; Mollenholt et al., 1994; Helmchen et al., 1995). BDNF has also been demonstrated to increase 5HT synthesis in raphe neurons in vitro by increasing expression of tryptophan hydroxylase (TPH; the rate-limiting enzyme for 5HT synthesis) (Eaton et al., 1995). Lumbar subarachnoid grafts of cells that synthesized both BDNF and 5HT were able to reverse chronic neuropathic pain after CCI (Eaton et al., 1997). Serotonergic inhibition of primary afferents (Yaksh and Wilson, 1979) and individual spinal neurons (Davies and Roberts, 1981), as well as the presence of ascending serotonergic sensory pathways (Anden et al., 1964), suggest that changes in the serotonergic systems might facilitate or inhibit the development of chronic pain after PNS and CNS injury (Besson, 1990). Activation of serotonin receptors and the tail-flick response (Millan, 1994) suggests that other non-serotonergic systems or their interaction with the descending serotonin system may play even a more important role in nociceptive responses.

It is unclear what therapeutic dose of BDNF may be required to affect neuropathic pain and how these levels of BDNF may affect sensory behaviors chronically in the absence of injury, since levels of BDNF secreted by 33BDNF.4 in vivo were not measured here. A group of animals which had no nerve injury but received 33BDNF.4 transplants did not demonstrate any changes in sensory behaviors (data not shown) after grafting, and partial nerve injury may require increased BDNF levels to reverse neuropathic pain. Studies of fibroblasts genetically engineered to synthesize and secrete BDNF (Galpern et al., 1996) have used ELISA methods to determine the rate of BDNF secretion in vitro at about 6 ng of BDNF/10^5 cells per day, and grafted cells were able to enhance dopamine function after chemical lesions of the substantia nigra. The 33BDNF.4 cells were transplanted when they were secreting about 26 ng/10^6 cells per day of the mature BDNF protein, but the differentiated 33BDNF.4 cells dropped their secretion rate to about 2.5 ng/day after 3 days in vitro. However, since this BDNF secreted from the 33BDNF.4 cells is bio-active (Fig. 4), even a 2–5 ng/day dose of BDNF should have significant in vivo effects. Many studies using exogenous BDNF, including that supplied by bio-engineered cells, used this order of magnitude of BDNF concentration for treatment (Liu et al., 1999; Shetty and Turner, 1999). Another issue of the in vivo effects of BDNF-secreting grafts besides a bio-active therapeutic dose is the time of transplant. In a recent study that looked at the effects of intrathecal GABA administration after CCI and reversal of neuropathic pain (Eaton et al., 1999a), the critical window of time where the behaviors could be reversed was from 1 to 3 weeks after CCI; later therapeutic intervention was ineffective. Other cell grafting studies (Eaton et al., 1997, 1999b) have used the same 1 week after CCI time point as used here. It is likely that even if the dose of BDNF supplied by the grafted 33BDNF.4 cells is low, its probable effects are to prevent the induction phase of neuropathic pain, e.g. probably by rescue of the loss of other endogenous inhibitory systems.

Cell grafts synthesizing both 5HT and BDNF (Eaton et al., 1997) that reversed neuropathic pain were also able to reverse the loss of endogenous GABA synthesis in the dorsal horn (Eaton et al., 1998) related to alteration in the expression of the GAD67 enzyme for GABA synthesis, suggesting a role for BDNF and the intrinsic GABA interneurons of the dorsal horn after nerve injury. BDNF has been demonstrated to directly rescue CNS GABA neurons and stimulate GABA neurotransmitter synthesis (Mizuno et al., 1994; Spenger et al., 1995; Arenas et al., 1996; Larkfors et al., 1996). The receptor trkB has been found on the GABAergic interneurons of the hippocampus (Zachrisson et al., 1996), suggesting that BDNF may directly modulate the function and/or survival of that cell type, such as the GABAergic interneurons of the dorsal horn. BDNF supports the survival of various embryonic neurons (Lindsay and Peters, 1984; Lindsay et al., 1985), prevents neuronal apoptosis in vivo due to excitotoxicity via down-regulation of

NMDA receptor subunit expression (Brandoli et al., 1998) and reversal of NMDA-induced inactivation of protein kinase C (Trembly et al., 1999) and prevents oxidative stress (Skaper et al., 1998) and Ca^{2+}-mediated events (Takei et al., 1999). Neuropathic pain following peripheral nerve injury, such as that seen after CCI (Bennett and Xie, 1988), appears to be subject to loss/down-regulation of GABAergic control and glutamate-mediated excitotoxicity (Kawamata and Omote, 1996; Mao et al., 1997). Intrathecal administration of the GABA-A receptor antagonist, bicuculline, caused a dose-dependent increase in the magnitude of hyperalgesia when given within a few days of CCI (Yamamoto and Yaksh, 1993). In a similar nerve injury model that includes the ligation of the L5/L6 nerve roots (Kim and Chung, 1992), IT injection of both the GABA-B agonist baclofen and the GABA-A agonist muscimol resulted in a dose-dependent antagonism of the induced tactile allodynia 2 weeks after injury (Hwang and Yaksh, 1997), suggesting that both GABA receptor types modulate spinal systems activated by low threshold mechanoreceptors which mediate tactile allodynia following peripheral nerve injury. Intrathecal administration of GABA within 1–3 weeks after CCI reversed the hyperalgesia and allodynia induced by the injury (Eaton et al., 1999a), suggesting the requirement for a critical level of GABA to prevent the induction of neuropathic pain.

The best common explanation for the changes in intraspinal biochemistry to account for the chronic pain that various peripheral and central injuries produce is the loss of inhibitory tone, or loss of modulation by the GABA-releasing interneurons in the cord after these various central and nerve injuries (Kjaer and Nielsen, 1983; Kingery et al., 1988; Yaksh, 1989; Hao et al., 1992; Zhang et al., 1994a; Stiller et al., 1995). Recent studies have demonstrated a concomitant down-regulation of a GABA-A receptor subunit mRNA in the DRG ipsilateral to 7 days following nerve injury (Fukuoka et al., 1998), suggesting a central plasticity and inhibition from a peripheral injury. Whether such loss of GABA synthesis is due to excitotoxic cell death of GABA interneurons or temporary or permanent alterations in the neurotransmitter's synthesis is not known, but neurotrophins such as BDNF may act directly to intervene in cell death and/or regulate GAD enzyme activity, as has been seen with BDNF and other GABA cells (Mizuno et al., 1994; Spenger et al., 1995; Arenas et al., 1996; Larkfors et al., 1996).

Alterations in spinal GABA after nerve injury supplied endogenously, either intrathecally (Eaton et al., 1999a) or via bio-engineered GABA cell grafts (Eaton et al., 1999b), potently reverse neuropathic pain, and BDNF supplied by 33BDNF.4 cell grafts may work by a similar mechanism, e.g. indirectly increasing spinal GABA levels and/or restoration of the endogenous inhibitory mechanisms.

There are significant issues surrounding the eventual development and use of human cell lines for cellular therapy, since these and other studies merely model the first steps in the use of immortalized cell grafts for pain. However, there are some interesting recent uses of human spontaneously-immortalized cell lines, for example the human NT2 cell line for stroke (Borlongan et al., 1998) and neurodegenerative diseases. Perhaps cell immortalization or precursor/stem cell approaches are a more realistic way to develop a useful source for cell therapy. With human (and other species) immortalization with SV40 Tag (Jha et al., 1998), the limit has been the requirement for a mitotic embryonic source. This technical gap is quickly narrowing with the development of smaller immortalization constructs that are less disruptive to the cell cycle, such as T155 (Truckenmiller et al., 1998), and the use of adeno-associated viral (AAV) infection (Bueler, 1999) of human cells for sequence incorporation. Also being developed is the use of Cre/lox disimmortalization for site direction recombination to remove the oncogenic Tag (Herman et al., 1997, 1999) before or after transplant. All such technologies are under development in my own laboratory (Eaton et al., 2000; Sagen and Eaton, 2000) to create human cell lines for cell therapy.

In summary, the objective of these studies was to use bio-engineered cell transplants that secrete the neurotrophin BDNF to alter the outcome of the behavioral effects of nerve injury that mimic the evoked hypersensitivity reported as painful in humans after similar nerve injuries. It is known that infusion of BDNF into the adult brain stem increases the synthesis of 5HT in endogenous raphe serotonergic neurons and provides potent analgesia (Martin-Iverson et al., 1994). Other cell transplants used after sciatic nerve constriction can potently reverse the hypersensitivity to thermal and tactile stimuli in the affected rat hindpaw, but the primary adrenal chromaffin cells currently being used for lumbar transplant in rat and human models for chronic pain secrete a variety of factors and it is difficult to assign the anti-nociceptive effects to a single agent produced by the cells. Cell lines engineered to synthesize a single factor, such as the neurotrophin BDNF, and used for cell therapy after nerve injury test various neurotransmitters, peptides and growth factors singly and specifically at doses that might not be expected to influence peripheral or supraspinal pain processing centers. Also, chronic delivery by cellular 'minipumps' to the dorsal lumbar cord controlling the sensory responses in the affected hindpaw is expected to eliminate problems associated with infusion pumps, i.e. lability of neurotrophins, infection at the catheter tip and an initial high dose of agents. Eventual use of cell therapy for chronic pain requires testing of each anti-nociceptive molecule secreted by engineered cells for its potential safety and efficacy in animal models.

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